Published in final edited form as:

Pancreas. 2012 March ; 41(2): 175–185. doi:10.1097/MPA.0b013e318227a6b7.

Proteomic analysis of formalin-fixed paraffin-embedded pancreatic tissue using liquid chromatography tandem mass spectrometry (LC-MS/MS)

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Abstract

Objectives—Formalin-fixed paraffin-embedded (FFPE) tissue is a standard method of specimen preservation for hospital pathology departments. FFPE tissue banks are a resource of histologically-characterized specimens for retrospective biomarker investigation. We aim to establish liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis of FFPE pancreatic tissue as a suitable strategy for the study of the pancreas proteome.

Methods—We investigated the proteomic profile of FFPE pancreatic tissue specimens, using LC-MS/MS, from 9 archived specimens that were histologically-classified as: normal (n=3), chronic pancreatitis (n=3), and pancreatic cancer (n=3).

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COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

JP carried out the experiments and drafted the original manuscript. JP and DC conceived of the study. JP, DC, and HS participated in its design and coordination. All authors assisted drafting the manuscript and approved the final manuscript.

Results—We identified 525 non-redundant proteins from 9 specimens. Implementing our filtering criteria, 78, 15, and 21 proteins were identified exclusively in normal, chronic pancreatitis, and pancreatic cancer specimens, respectively. Several proteins were identified exclusively in specimens with no pancreatic disease: *spink1*, retinol dehydrogenase, and common pancreatic enzymes. Similarly, proteins were identified exclusively in chronic pancreatitis specimens: collagen $\alpha 1$ (XIV), filamin A, collagen $\alpha 3$ (VI), and *SNC73*. Proteins identified exclusively in pancreatic cancer included: annexin 4A and fibronectin.

Conclusions—We report that differentially-expressed proteins can be identified among FFPE tissues specimens originating from individuals with different pancreatic histologies. The mass spectrometry-based methodology used herein has the potential to enhance biomarker discovery and chronic pancreatitis research.

Keywords

pancreas; pancreas tissue; biomarker; chronic pancreatitis; FFPE; pancreatic cancer

Introduction

Diseases of the pancreas affect greater than 1 million persons in the United States annually, resulting in nearly \$3 billion in direct and indirect medical costs ¹. The development of a better understanding of the biomolecular mechanisms of pancreatic diseases, such as chronic pancreatitis and pancreatic cancer, represents an important step in treatment aimed to modify or retard disease progression before end-stage disease complications occur.

Organ-specific biomarker discovery studies often analyze easily obtainable body fluids, such as serum and urine ^{2–5}. Because systemic fluids are representative of the entire body, they are more prone to confounding factors resulting from the effects of other diseases. In addition, low abundance biomarkers may be vastly diluted among other serum and urine proteins. One alternative strategy is to study proximal body fluids, which bathe the organ of interest. Due to the vast disparity by which these specimens are collected, stored, and processed, meaningful inter-laboratory data comparisons have been limited in scope. Recent efforts have been made to standardize specimen collection, handling, and storage of various body fluids ^{2, 3, 6–12}. However, analogous methodologies for optimized proteomic analysis of archived pathology specimens have yet to be established.

The gold-standard of specimen preservation for hospital pathology departments is formalin-fixed paraffin-embedded (FFPE) tissue. FFPE tissue banks offer a vast resource for retrospective protein biomarker investigation. Such specimens have historically been used to investigate the cellular localization of specific proteins via immunohistochemistry (IHC) ¹³. However, IHC requires *a priori* knowledge of proteins to be specifically targeted and this technique is not suitable for large-scale protein identification. The application of state-of-the-art proteomics techniques to such tissue repositories may enhance our understanding of the biomolecular mechanisms of disease in a particular organ.

Mass-spectrometry-based proteomics is quickly becoming the quintessential strategy for unbiased large-scale protein investigation ¹⁴. Archived FFPE pancreatic tissue specimens offer an alternative method to investigate biochemical pathways and to discover biomarkers of pancreatic disease. Analogous techniques have been applied previously to FFPE tissues from various organs ¹⁵. A recent study has investigated pancreatic cancer FFPE tissue using a Liquid Tissue workflow for a global proteomics analysis ¹⁶. However, to our knowledge, we report the first comparison of normal, chronic pancreatitis, and pancreatic cancer FFPE pancreas tissue.

As a proof of principle, we compare the proteomic profile of FFPE pancreatic tissue specimens histologically-classified as: a) no pancreatic disease, b) chronic pancreatitis, and c) pancreatic cancer.

The major objectives of this proteomic investigation are:

1. prepare FFPE pancreatic tissue for proteomic analysis,
2. identify proteins present in FFPE pancreatic tissue using liquid chromatography-coupled with tandem mass spectrometry (LC-MS/MS),
3. compare the protein profiles isolated from the aforementioned specimens, and
4. investigate differences in characteristics (e.g., localization, function, and biological pathways) of the proteins across cohorts.

We aim to establish LC-MS/MS analysis of FFPE pancreatic tissue as a viable strategy for the study of the pancreas proteome.

Materials and Methods

Study design and setting

Proteomic analysis of archived formalin-fixed paraffin-embedded (FFPE) pancreas tissue in an academic center.

Study population

This protocol was approved by the Institutional Review Board at Brigham and Women's Hospital (IRB # 2007-P-002480/1).

Materials

Heptane (product #51750) was purchased from Sigma-Aldrich. Pep-clean C18 spin columns (product # 89870) were purchased from Thermo Scientific (Waltham, MA). Other reagents and solvents were from Sigma-Aldrich (St. Louis, MO) and Burdick & Jackson (Morristown, NJ), respectively.

Formalin fixing and paraffin embedded of pancreatic tissue

FFPE tissue specimens were fixed in 10% neutral buffered formalin for 48 h (Dana-Farber/Harvard Cancer Center research pathology core). Routine paraffin treatment was as follows: Specimens were treated with 70% ethanol for 45 min twice, 80% ethanol for 60 min twice, 95% ethanol for 60 min twice, 100% ethanol for 60 min three times, followed by #83 Xylene substitute for 60 min twice and paraffin for 60 min twice. For tissue microdissection, 5- μ m-thick tissue sections were cut from the FFPE whole-mount pancreatic tissue block, mounted on standard glass slides, and heated for 60 min at 60°C. Slides were stored at 27°C until use.

Experimental workflow

The overall experimental analysis was shown in Figure 1: 1) FFPE tissue specimens were obtained from the Dana-Farber/Harvard Cancer Center research pathology core, 2) a 1.5 cm $\times$ 1 cm $\times$ 5 μ m slice of the FFPE tissue was scraped into a microcentrifuge tube, 3) paraffin was removed with heptane, 4) disulfide bonds were reduced with dithiotreitol (DTT) and alkylated with iodoacetamide, 5) sample was digested overnight with trypsin, 6) peptides were isolated with Pep-clean C18 spin columns, 7) LC-MS/MS analysis was carried out, and 8) data was searched against a protein database after which bioinformatics processing was performed.

Sample preparation for mass spectrometry analysis

The sample preparation protocol for mass spectrometry analysis was assembled from several sources^{15, 17–20}. After removing the excess paraffin, a 1.5 cm × 1 cm square of the 5 μm thick FFPE pancreatic tissue specimen from each glass slide was scraped using a clean razor blade into a 2 mL microcentrifuge tube. To remove the remaining paraffin, 0.5 mL heptane was added to each sample, followed by vigorous vortexing for 10 seconds and incubation at room temperature for 1 h. After the incubation, 25 μL of methanol was added to each tube, followed by vigorous vortexing for 10 seconds and centrifugation (20,000 g for 2 min at 4°C). Immediately following centrifugation, the upper (heptane) layer was discarded and the lower layer was allowed to evaporate. To extract proteins from this mixture, the dried material was resuspended in 250 μL 6M guanidine-HCl/50 mM ammonium bicarbonate/20 mM DTT, pH 8.5, briefly sonicated, and incubated at 70°C for 1 h. After cooling, iodoacetamide was added to a final concentration of 40 mM and the sample was incubated at room temperature in the dark for 1 h. The alkylation reaction was quenched by adding 3 μL of 2 M DTT. For tryptic digestion, the sample was diluted 1:6 with 50 mM ammonium bicarbonate pH 8.1 to reduce the concentration of guanidine-HCl to 1 M. Each sample was incubated with 2.5 μg of trypsin. Following the incubation, the reaction was acidified with formic acid to a final concentration of 0.1% and evaporated by vacuum centrifugation. To remove interfering substances prior to mass spectrometry, peptides were isolated with Pep-clean C18 spin columns using manufacturer's instructions. Samples were vacuum centrifuged to dryness and stored at –80°C until analysis. Immediately prior to analysis, the peptides were resuspended in sample loading buffer (5% formic acid, 5% acetonitrile, 90% water).

Mass spectrometry

Mass spectrometry analysis was performed at the Proteomics Center at Children's Hospital Boston. The resuspended peptides were subjected to peptide fractionation using reversed-phase high pressure liquid chromatography (HPLC; Thermo Scientific) and the gradient-eluted peptides were analyzed using an LTQ FT Ultra mass spectrometer (Thermo Scientific, Waltham, MA). The liquid chromatography columns (15 cm × 100 μm ID) were packed in-house with Magic C18 (5 μm, 100 Å, Michrom BioResources, Auburn, CA), into PicoTips (New Objective, Woburn, MA). Samples were analyzed with a 90 min linear gradient (0–35% acetonitrile with 0.2% formic acid) and data were acquired in a data-dependent manner, in which MS/MS fragmentation was performed on the six most intense peaks of every full MS scan.

Bioinformatics and data analysis

All data generated from the gel sections were searched against the international protein index (IPI) human database (v3.61) using the Paragon Algorithm²¹, which is integrated into the ProteinPilot search engine (v.3; AB SCIEX, Foster City, CA). Search parameters were set as follows: sample type, identification; Cys alkylation, iodoacetamide; Instrument, Orbitrap/FT (1–3 ppm); special factors, none; ID focus, none; database, IPI- human (v.3.61); detection protein threshold, 99.0%; and search effort, thorough ID. Thus, using our stringent criteria, we defined an identified protein as one with 99% confidence, as determined by the Paragon Algorithm²¹. For purpose of comparative proteome investigation, a protein was required to have been identified in 2 of 3 specimens of a particular cohort.

Gene ontology (GO) analysis

GO analysis²² was performed using the GoFact online tool^{23, 24} and verified manually using data from the UniProt²⁵ database. Annotation categories used were as follows: *functions*: enzyme regulator activity, ion binding, kinase activity, lipid binding, nucleic acid

binding, nucleotide binding, oxygen binding, peptidase activity, protein binding, signal transducer activity, structural molecule activity, transcription regulator activity, and transporter activity; *cellular component*: cytoskeleton, cytosol, endoplasmic reticulum (ER), endosome, extracellular matrix, extracellular region, golgi apparatus, lysosomes, membrane, mitochondrion, nucleus, ribonucleoprotein complex, and vacuole.

KEGG pathway analysis

Using the DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Database (<http://david.abcc.ncifcrf.gov/>) interface^{26, 27}, we analyzed our protein lists with KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis^{28–30}.

Results

Proteomic analysis of FFPE tissue identified several hundred proteins using mass spectrometry-based proteomic techniques

FFPE tissue from nine histologically-classified archived specimens [no pancreatic disease (n=3), chronic pancreatitis (n=3) and pancreatic cancer (n=3)] were subjected to mass spectrometry-based proteomic analysis, as is depicted in Figure 1. For the analysis of each specimen, we used a single slice of FFPE tissue with dimensions of 1.5 cm × 1 cm × 5 μm. Mass spectrometric analysis of all 9 FFPE specimens identified a total 525 non-redundant proteins, which we list in Supplementary Table 1. A non-redundant protein is one that has been counted only once, regardless of the number of specimens in which it has been identified.

Of the proteins identified, some proteins were exclusive to a particular cohort

Table 1 summarizes the number of non-redundant proteins identified in this study. Taking each specimen individually, we identified 266, 102, and 199 non-redundant proteins in each normal pancreas (NP) specimen. Similarly, we identified 82, 98, and 85 non-redundant proteins in each chronic pancreatitis (CP) specimen. Likewise, we identified 130, 183, and 134 non-redundant proteins in each pancreatic cancer (PC) specimen. We then merged the three protein lists for each cohort to determine the number of non-redundant proteins identified *within* each cohort, as listed in Table 1. After merging these lists, the numbers of non-redundant proteins were: 312 in normal pancreas, 162 in chronic pancreatitis, and 178 pancreatic cancer. To minimize false positives, we tightened our analysis criteria by using a filtering strategy which included only proteins that were identified in 2 of 3 specimens. This filtering reduced the number of identified non-redundant proteins to 162 in normal pancreas, 62 in chronic pancreatitis, and 101 pancreatic cancer.

We determined the proteins that were exclusive to each cohort, in other words, proteins that appeared exclusively in one particular cohort and neither of the other cohorts. Thus, we identified 78 proteins in normal pancreas (Table 2), 15 proteins in chronic pancreatitis (Table 3), and 21 proteins in pancreatic cancer (Table 4) specimens which were exclusive to each particular cohort. These values were visualized by a Venn diagram in Figure 2. Among those proteins identified exclusively in the normal pancreas specimens include: carboxypeptidase B, carboxy ester lipase 2, retinal dehydrogenase 1, and spink1. Similarly, collagen α1 (XIV), collagen α3 (VI), filamin A, and SNC73 were identified exclusively in the chronic pancreatitis specimens. In addition, annexin 4A and fibronectin were among the proteins that were identified exclusively in the pancreatic cancer specimens.

Gene ontology (GO) analysis among the proteins identified from each cohort revealed moderate differences

As an initial investigation of the proteins which we have identified in FFPE tissue, we examined the subcellular localization (Figure 3A) and molecular function (Figure 3B) of the proteins in each of the three cohorts. For this analysis, we used the total proteins identified in each specimen (312 for normal, 162 for chronic pancreatitis and 178 for pancreatic cancer). In terms of subcellular localization, normal and pancreatic cancer specimens were similar, however there were striking differences with the chronic pancreatitis cohort. The chronic pancreatitis cohort had a 1.5-fold or greater number of proteins classified as cytoskeletal, or originating from the extracellular matrix, extracellular region (secreted), or nucleus. Conversely, there was a 1.5-fold or lower number of proteins which are cytosolic, originate from endoplasmic reticulum or are ribonucleic proteins when compared to the other two cohorts. Similarly, when examining the molecular functions of the proteins that we identified, there was a 1.5-fold or greater increase in the number of structural and transporter proteins, and a 1.5-fold or lower number of proteins involved in nucleic acid binding or proteolysis when comparing the chronic pancreatitis specimens with those from the other two cohorts.

KEGG pathway analysis

As a preliminary analysis of the biomolecular pathways in which the proteins we have identified in FFPE tissue were involved, we examined the proteins exclusive to each of the three cohorts using KEGG pathway analysis. Our data revealed that several proteins appearing exclusively in the normal pancreas specimens were involved in ECM-receptor interactions. These proteins included agrin, laminin $\alpha 2$, laminin $\alpha 5$, and vitronectin. In addition, there were several proteins involved in ribosomal function. In the chronic pancreatitis specimens, KEGG analysis revealed that collagen type IV $\alpha 3$ and filamin A were involved in the focal adhesion pathway. In pancreatic cancer specimens, KEGG analysis revealed involvement in the regulation of actin cytoskeleton and focal adhesion. The proteins involved in regulation of actin cytoskeleton included fibronectin 1, myosin 9, viculin, and actin gamma 1; and those involved in focal adhesions included actin gamma 1, collagen type I $\alpha 2$, vinculin, and fibronectin 1.

Supplementary data

We included several supplementary tables listing the identified proteins. The 525 non-redundant proteins from the 9 specimens were listed in Supplementary Table 1 including those identified only in one specimen. We also included several tables listing proteins which overlap between two cohorts. Supplementary Table 2 lists the 10 non-redundant proteins which were determined to be in common between normal and chronic pancreatitis. Supplementary Table 3 lists the 43 non-redundant proteins in common between normal and pancreatic cancer. Supplementary Table 4 lists the 6 non-redundant proteins in common between chronic pancreatitis and pancreatic cancer. In the tables, the percent protein coverage (%cov) and number of identified peptides (#pep) were listed. The percent coverage referred to the percentage of amino acid residues of a particular protein that was present in identified peptides for that particular protein. The number of peptides referred to the number of non-redundant peptides that were used to identify that particular protein. Supplementary Table 5 lists the proteins appearing in 2 of 3 specimens of every cohort. Ten proteins appeared in all nine FFPE specimens, these are: Fibrillin-1, hemoglobin subunits α and β , histone H11.4, histone H4, heat shock protein $\beta 1$, trypsin-1, ribosomal protein L19, peptidylpropyl isomerase A-like protein, and vimentin. Such consistently-identified proteins can serve to define a core set of proteins that are identified from FFPE pancreatic tissues.

Discussion

We have successfully applied LC-MS/MS analysis to FFPE tissue. In total, we have identified 525 non-redundant proteins among all 9 specimens. We used a filtering strategy that required a protein to appear in 2 of 3 specimens to be included in subsequent analyses. By comparing these proteins, we have identified those that were found exclusively in normal (78), chronic pancreatitis (15), and pancreatic cancer (21). The FFPE tissue processing techniques and mass spectrometry strategies which we perform herein with pancreatic tissue have been used previously to study tissues of other organ systems, including prostate³¹, cochlea¹⁹, liver^{18,32}, glioblastoma³³, renal carcinoma³⁴, kidney glomeruli³⁵, mesenchymal tissue¹⁷, and B-cell lymphoma cells³⁶. A previously published study analyzed pancreatic cancer FFPE tissue using a Liquid Tissue workflow for a global proteomics analysis¹⁶. We, however, present the first comparative LC-MS/MS analysis of FFPE tissue from a wide spectrum of pancreatic disease that included: normal, chronic pancreatitis, and pancreatic cancer FFPE tissue.

Our mass spectrometry-based analysis revealed several proteins which are exclusive to specimens from particular cohorts. Below, we select several proteins which may merit further investigation to verify their role in pancreatic cancer or chronic pancreatitis. Proteins identified exclusively in the normal pancreas specimens (Table 2) include:

Carboxypeptidase B, carboxy ester lipase 2, retinal dehydrogenase 1, and spink1.

Carboxypeptidase B and carboxy ester lipase 2 are common pancreatic enzymes, and are expected to be present in healthy pancreata³⁷. Retinal dehydrogenase 1 binds free retinal and cellular retinol-binding protein-bound retinal and converts retinaldehyde to retinoic acid, thus having a role in vitamin A metabolism³⁸. Coincidentally loss of stored cytoplasmic vitamin A is related to pancreatic stellate cell activation and fibrosis associated with chronic pancreatitis³⁹. Spink 1 is a pancreatic secretory trypsin inhibitor which prevents the trypsin-catalyzed premature activation of zymogens within the pancreas. Defects in the gene encoding the spink 1 protein has been widely studied in pancreatitis and pancreatic cancer^{40–43}. In addition to the abundance of proteins involved with pancreatic enzymes, KEGG pathway analysis revealed proteins, agrin, laminin α 2, laminin α 5, and vitronectin, found exclusively in the normal specimens as having roles in extracellular matrix receptor interactions. Such interactions may be essential in the turnover of extracellular matrix, a process which is impaired in chronic pancreatitis and pancreatic cancer patients.

Selected proteins of interest identified exclusively in the chronic pancreatitis specimens (Table 3) include: Collagen α 1 (XIV), collagen α 3 (VI), filamin A, and SNC73. Collagen α 1 (XIV), a putative structural molecule that associates with collagen fibrils, may be involved in the rearrangement of connective tissue. Similarly, collagen α 3 (VI) is an extracellular matrix structural constituent involved in the TGF- β receptor signaling pathway, which is implicated in pancreatic fibrosis. Also, filamin A, acts in actin cytoskeleton organization and apoptosis, regulates cell migration and plays a role in disrupting the cellular cytoskeleton⁴⁴. SNC73, a protein that shares homology with immunoglobulins, has been found to be a potential biomarker of colorectal cancer⁴⁵. These secreted or extracellular matrix proteins may have roles in the inflammatory cascade that is thought to lead to pancreatic fibrosis^{46, 47}. Similarly, KEGG pathway analysis has identified extracellular proteins, collagen type I α 2 and filamin A, which are involved in focal adhesions, and may have an analogous role in pancreatic cancer development.

Selected proteins of interest identified exclusively in pancreatic cancer (Table 4) include: annexin 4A and fibronectin. Annexin A4 is a calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis. In the pancreas, this protein has a role in binding zymogen granules, which may have implications in proper proenzyme export

from the pancreas^{48–50}. Another structural protein, fibronectin binds cell surfaces and various compounds including collagen, fibrin, heparin, DNA, and actin⁵¹. The inhibition of fibronectin-dependent proliferation has recently been shown to have antitumor effects⁵². In addition, annexin A5 and 14-3-3zeta were identified in both chronic pancreatitis and pancreatic cancer specimens (Supplementary Table 3). Both annexin A5^{53, 54} and 14-3-3zeta⁴⁹ have been associated with pancreatic cancer in previously published proteomic studies. Also, annexin A5 has been found by DNA microarrays to be a biomarker of pancreatic cancer⁵⁵. In addition, we have determined using KEGG pathway analysis that proteins in pancreatic cancer specimens, fibronectin 1, myosin 9, viculin, collagen type I $\alpha 2$, vinculin, fibronectin 1, and actin gamma 1, were mapped to focal adhesion and/or actin cytoskeletal pathways. Studies have shown an integral link between focal adhesion and/or actin cytoskeletal proteins and pancreatic cancer^{56–60}. Further biochemical studies, such as western blotting or immunoprecipitation must be performed to analyze further the identified proteins in terms of their GO annotations and KEGG pathways.

Although we were able to identify a total of 525 proteins, harsh FFPE tissue preparation conditions presents a barrier which limits proteomic protein identification. Additionally, there is an inherent difficulty in extracting intact proteins from FFPE tissue due to protein crosslinking that is associated with specimen preparation. Chemically, during tissue fixation, formalin adds a methylene hydrate group to the side chain of certain amino acids leading to a methylene bridge formation that results in protein crosslinking^{61, 62}. Although modifications are common on side chain moieties of lysyl, arginyl, tyrosyl, histidyl and seryl⁶³, all 20 amino acids have been successfully identified during LC-MS/MS analysis of peptides originating from FFPE tissue with frequencies similar to that in frozen tissue^{18, 32}. However, formalin-induced intra- and intermolecular crosslinking hinders the solubility of proteins, often complicating the extraction of proteins, and potentially larger peptides, from FFPE specimens⁶⁴. In fact, harsh formalin fixation (greater than 96 hours) has been shown to result in DNA fragmentation and enhanced protein crosslinks which have adverse effects on genomic and proteomic investigations⁶⁵. Analogously, in transcriptomics analysis, the extraction of mRNA is also significantly reduced as a result of extensive periods of fixation⁶⁶. Future improvements in the methods of sample analysis may overcome these difficulties, which would greatly increase the depth of the proteomic analysis of preserved historical FFPE specimens.

Fresh frozen tissue presents an alternative specimen preservation strategy for proteomic analyses. However, frozen tissue is difficult to store and requires liquid nitrogen or ultra-low temperature (-80°C) freezer storage. In contrast to frozen specimens, FFPE specimens are generally processed with standard methodologies and are inherently stable at room temperature. Unlike FFPE tissue, fresh-frozen tissue has been shown to not properly maintain its morphology^{63, 67}. Furthermore, other recent studies in tissues from follicular lymphoma¹⁵, ear canal¹⁹, renal carcinoma³⁴, colon and breast cancer⁶⁸, and liver²⁰, have identified nearly as many, or even more proteins using FFPE tissue than frozen tissue. A recent study by Ostasiewicz, *et al.*, has shown that not only is the proteome preserved in FFPE mouse liver tissue, but so are the posttranslational modifications of phosphorylation and N-glycosylation³². Although several studies have compared proteins identified from fresh-frozen and FFPE tissue specimens, no such comparison has been performed using pancreatic tissue. While there may be proteolytic enzymes in previously-studied tissues, pancreatic tissue has a higher propensity of self-digestion as a result of endogenous proteases. A systematic study of a statistically significant number of FFPE and frozen pancreatic tissue is needed to further assess the benefits of one method over the other for mass spectrometry-based proteomic analyses. As such, assaying the benefits of modifications to current strategies, including, but not limited to, the addition of protease

inhibitors, fixation at lower temperatures, and meticulous handling of tissue on ice, may allow for a greater depth of proteomic analysis from FFPE tissue of pancreatic origin.

As with other mass spectrometry-based proteomics studies, sample complexity may limit the analytical depth of any proteomic study. Many recent studies have used various forms of fractionation to minimize the complexity of the proteome in the FFPE tissue specimen under investigation, which may increase the number of identified proteins. In general, decreases in protein complexity can be achieved by fractionation at the peptide, protein, subcellular, or cellular level prior to mass spectrometric analysis. In our previous studies, we have investigated pancreatic and gastroduodenal fluid using GeLC-MS/MS techniques, which fractionates by electrophoretic mobility^{11, 69, 70}. Although protein level fractionation may be thought of as impractical for FFPE tissue, as intermolecular crosslinking can impair protein extraction and mobility during fractionation, several research groups have recently been successful in SDS-PAGE analysis of whole proteins extracted from FFPE tissues^{34, 68, 71, 72}. Alternatively, commonly-used methods of peptide fractionation include various forms of liquid chromatography and separation by isoelectric points⁷³. More recently, a novel sample preparation strategy, FASP (Filter Aided Sample Preparation) has been developed and used successfully to analyze samples which are difficult to process including membrane proteins and FFPE tissue specimens^{74, 75, 32}. FFPE tissue fractionation can also be performed at the cellular level with the isolation of specific cell types or regions of tissue, as was previously done for pancreatic cancer tissue analysis¹⁶. Similar to tissue biopsies, FFPE sections generally consist of different cell types. If the aim is to target only particular cell types, serial sectioning and laser-capture microdissection (LCM) is suitable for such analyses^{76, 77, 35}. Such cellular fractionation by microdissection decreases sample complexity and thus increases analytical depth. In terms of future investigations of FFPE pancreatic tissue, it may be practical to utilize a similar methodology to enrich for regions rich in ductal or acinar cells via microdissection.

A major advantage of pursuing the proteomic investigation of FFPE tissue is the availability of a vast archive of patient samples with clearly defined patient histories and well-documented diagnoses. Such specimens are an invaluable resource for translational studies, enabling the analysis of pancreatic tissue specimens from patients with known outcomes. The value of these tissues is not limited to biomarker discovery, but also may result in a better understanding of the molecular processes and pathways that lead to end-stage disease. In addition, the use of proteomics for FFPE tissue analysis allows for correlation of these data with miRNA and/or mRNA studies that use FFPE tissue^{78–81}. Challenges which must be addressed, however, include the establishment of more efficient protein or peptide extraction and the assessment of potential modifications of the amino acid residues resulting from specimen preparation.

In summary, we have shown that mass spectrometry-based FFPE tissue analysis is applicable to the study of pancreatic tissue. Our results indicate that differentially-expressed proteins can be identified among FFPE tissues specimens originating from individuals with normal pancreata, chronic pancreatitis, and pancreatic cancer. With further methodological optimization, LC-MS/MS-based proteomic analysis of FFPE pancreatic tissue specimens may provide novel protein targets for improved therapy or a means of high-throughput validation of current biomarkers. Given the vast availability of FFPE specimens, large databases of diseased and normal tissue-specific proteins may be assembled for validation via orthogonal methodologies, such as immunohistochemistry studies. The work which we present herein demonstrates the potential use of LC-MS/MS for the analysis of archived FFPE pancreatic tissue and provides a basis upon which biomarker studies can be developed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funds were provided by the following NIH grants: 1 F32 DK085835-01A1 (JP), 1 R21 DK081703-01A2 (DC) and 5 P30 DK034854-24 (Harvard Digestive Diseases Center; DC). In addition, we would like to thank the Burrill family for their generous support through the Burrill Research Grant. We would also like to thank members of the Steen Lab at Children's Hospital Boston, in particular John FK Sauld and Dominic Winter, as well as Kate Repas (Research Manager) from the Center for Pancreatic Disease at Brigham and Women's Hospital for their technical assistance and critical reading of the manuscript.

LIST OF ABBREVIATIONS

CP	chronic pancreatitis
FFPE	formalin-fixed paraffin-embedded
GO	gene ontology
KEGG	Kyoto Encyclopedia of Gene and Genomes
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
LCM	laser capture microdissection
NP	normal pancreas
PC	pancreatic cancer

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Figure 1. Experimental workflow

1) FFPE tissue specimens were obtained, 2) a 1.5 cm × 1 cm × 5 μm slice of sample was scraped into a tube, 3) paraffin was removed with heptane, 4) disulfide bonds were reduced with dithiotreitol (DTT) and alkylated with iodoacetamide, 5) sample was digested overnight with trypsin, 6) peptides were isolated, 7) LC-MS/MS analysis was performed, and 8) bioinformatics analysis was performed.

Figure 2. Overlap of proteins among the three cohorts

Venn diagrams showing unique and overlapping proteins among the three cohorts. Also indicated in the figure are the tables in which these sets of proteins are listed. Abbreviations: NP, normal pancreas; CP, chronic pancreatitis; PC, pancreatic cancer.

A)

B)

Figure 3. Gene ontology annotation

A) Subcellular localization. B) Molecular function. We include in this analysis all non-redundant proteins that were identified in each cohort. Abbreviations: NP, normal pancreas; CP, chronic pancreatitis; PC, pancreatic cancer.

Table 1
Summary of the number of proteins identified from FFPE tissue specimens by cohort.

cohort	specimen #	number of non-redundant identified proteins		
		total*	within each cohort	in 2 of 3 specimens
NP	1	266		
	2	102	312	162
	3	199		78
CP	1	82		
	2	98	162	62
	3	85		15
PC	1	130		
	2	183	178	101
	3	134		21

Abbreviations: NP, normal pancreas; CP, chronic pancreatitis; PC, pancreatic cancer.

* A total of 525 non-redundant proteins were identified in the study.

Table 2

Proteins (78) identified in at 2 of 3 normal pancreas specimens and not in the other two cohorts, as illustrated in the Venn diagram in Figure 2. The number of peptides (#pep) identified and the sequence coverage (%cov) of each protein are indicated.

Name	IP1 #	NP1		NP2		NP3	
		#pep	%cov	#pep	%cov	#pep	%cov
19 kDa protein	IP100795717	5	59.4			3	45.5
21 kDa protein	IP100478208	4	43.5			3	46.2
ACTN4 actinin-4	IP100013808	3	13.8			2	9.8
ADH1B cDNA FLJ51682, similar to Alcohol dehydrogenase 1B	IP100872991	1	10.8	2	14.6		
AGRN Agrin	IP100374563 *	2	15.6	1	5.8	2	7.3
ALDH1A1 Retinal dehydrogenase 1	IP100218914	3	20.2			4	16.6
ANXA4 annexin IV	IP100793199	12	40.5			8	40.8
ATP1A1 Na ⁺ /K ⁺ -ATPase α 1 subunit isoform d	IP100930085	4	10.3			2	6.8
cDNA FLJ50378, Phenylalanyl-tRNA synthetase α chain	IP100909657	2	11.1			2	15.1
cDNA FLJ56074, 150 kDa oxygen-regulated protein	IP100922838	2	15.1			1	10.9
cDNA FLJ60461, similar to Peroxiredoxin-2	IP100909207	3	28.4			3	31.7
CEL carboxyl ester lipase (bile salt-stimulated lipase) isoform 2	IP100936498	9	28.6			3	13.8
CELA3B 33 kDa protein	IP100871512			2	10.7	6	33.6
CKAP4 Isoform 1 of Cytoskeleton-associated protein 4	IP100141318	3	16.8			3	22.1
CLTC Isoform 2 of Clathrin heavy chain 1	IP100455383	3	10.6			2	7.6
CPB1 Carboxypeptidase B	IP100009826	6	40.5			6	33.3
DCN Isoform A of Decorin	IP100012119 *	3	30.9	6	24.5	1	29
DDT 14 kDa protein	IP100878984	3	24.2			3	39.4
FGG 50 kDa protein	IP100877792			1	22.9	3	19.1
GDI2 GDP dissociation inhibitor 2 isoform 2	IP100640006	2	18.8			2	32
GP2 cDNA, FLJ92575, glycoprotein 2, (GP2)	IP100937053	2	17.2			6	14.5
HIST2H2AC Histone H2A type 2-C	IP100339274	9	78.3	5	58.1		
HNRNPA1 A1-A of nuclear ribonucleoprotein A1	IP100465365	3	24.4			3	29.4
HSPA8 Isoform 1 of Heat shock cognate 71 kDa protein	IP100003865	6	30.3			5	13.8
HSPB6 Heat shock protein β 6	IP100022433	2	29.1	1	21.1		
HSPD1 60 kDa heat shock protein	IP100784154	11	31.9			3	20.1

Name	IP1 #	NP1		NP2		NP3	
		#pep	%cov	#pep	%cov	#pep	%cov
IGHA1 cDNA, similar to Ig α1 chain C region	IP100449920			3	37.9	4	35.9
INS;INS-IGF2;IGF2 Insulin	IP100001508			12	76.4	3	75.5
LAMA2 Putative uncharacterized protein LAMA2	IP100873889	3	6.4			1	7.1
LAMA5 Laminin subunit α5	IP100783665	4	8.3	2	6.4		
LMNA Isoform A of Lamin-A/C	IP100021405	3	19.6			3	13.7
LRRCA7 Leucine-rich repeat-containing protein 47	IP100170935	2	9.9			2	7.4
MDH2 cDNA FLJ52880, Malate dehydrogenase	IP100924593	7	50.3			1	15.5
MYL6 cDNA FLJ56329, similar to Myosin light polypeptide 6	IP100796366			3	36.6	4	27.3
PARK7 Protein DJ-1	IP100298547	3	48.2			1	33.3
PEBP1 Phosphatidylethanolamine-binding protein 1	IP100219446	2	50.3			2	50.8
PHGDH D-3-phosphoglycerate dehydrogenase	IP100011200	6	38.5			4	26.5
PKM2 Isoform M1 of Pyruvate kinase isozymes M1/M2	IP100220644	6	29.9	4	35.2		
PRDX4 Peroxiredoxin-4	IP100011937	4	24.7			3	17.7
PRELP Prolargin	IP100020987	3	18.1	2	21.7		
PRSS1 PRSS1 protein	IP100815665	15	33.6			20	33.6
RCTP1;TP11 Isoform 1 of Triosephosphate isomerase	IP100797270 *	9	52.2	2	45.8	6	46.6
REG1B Lithostathine-1-beta	IP100009197	14	49.4			1	13.9
RPL10A 60S ribosomal protein L10a	IP100412579	2	27.7			2	22.1
RPL18A 60S ribosomal protein L18a	IP100026202	5	61.4			6	56.3
RPL21P16;RPL21P19;RPL21 60S ribosomal protein L21	IP100247583	9	63.1			5	62.5
RPL23 Similar to ribosomal protein L23	IP100795408	7	53.6			6	55.7
RPL26 60S ribosomal protein L26	IP100027270	3	37.9			2	32.4
RPL3 60S ribosomal protein L3	IP100550021	6	34			5	31.3
RPL30 Putative uncharacterized protein RPL30	IP100872940	3	67.8			1	28.7
RPL35A 60S ribosomal protein L35a	IP100029731	3	84.6			4	41.8
RPL36 60S ribosomal protein L36	IP100216237	4	51.4			3	44.8
RPL37A 60S ribosomal protein L37a	IP100414860	2	69.6			4	66.3
RPL38 8 kDa protein	IP100792410	3	22.4			4	50.8
RPL4 cDNA FLJ50996, similar to 60S ribosomal protein L4	IP100795303			4	41.6	11	69.3
RPL7A 60S ribosomal protein L7a	IP100299573	8	54.1			6	47

Name	IP1 #	NP1		NP2		NP3	
		#pep	%cov	#pep	%cov	#pep	%cov
RPN1 Dolichyl-diphosphooligosaccharide--protein glycosyltransferase	IP100025874	4	12.7			5	13.2
RPS10P29 similar to ribosomal protein S10	IP100398673	2	25.7			2	36.3
RPS12 40S ribosomal protein S12	IP100013917	7	45.5			3	27.3
RPS15 40S ribosomal protein S15	IP100479058	4	51			1	16.6
RPS18 40S ribosomal protein S18	IP100013296	4	54.6			4	30.9
RPS28 40S ribosomal protein S28	IP100719622	5	56.5			3	69.6
RPS3 40S ribosomal protein S3	IP100011253	3	23.9			4	32.1
RPS3A 40S ribosomal protein S3a	IP100419880	5	47.7			2	22.4
RPS5 40S ribosomal protein S5	IP100008433	4	48.5			5	34.8
RPS7P10 similar to mCG19129	IP100887413	3	33			2	43.5
SERPINB1 Leukocyte elastase inhibitor	IP100027444	2	8.4			6	23.8
SFPQ Isoform Long of Splicing factor, proline- and glutamine-rich	IP100010740	2	23.6			1	9.8
SNORA67:EIF4A1 46 kDa protein	IP100871852	2	21.9			1	16.5
SPINK1 Pancreatic secretory trypsin inhibitor	IP100020687	1	43			2	60.8
SPR Sepiapterin reductase	IP100017469	1	17.6			1	15.3
SSR4 Protein	IP100646864	1	13.3			2	20.8
TUBA1B Tubulin α 1B chain	IP100930688	4	19.5	2	17.1		
TUBB2C Tubulin β 2C chain	IP100007752	7	27.6	7	31		
UBA1 Ubiquitin-like modifier-activating enzyme 1	IP100645078	5	14.6			2	12.3
VTN Vitronectin	IP100298971	3	10.3	1	19.9		
YWHAH Isoform Long of 14-3-3 protein beta/alpha	IP100216318	4	35.8			4	27.2
YWHAH cDNA FLJ51975, moderately similar to 14-3-3 protein epsilon	IP100795516	3	44.9			3	24.3

Abbreviations: NP, normal pancreas; CP, chronic pancreatitis; PC, pancreatic cancer.

* denotes proteins appearing in all 3 specimens.

Proteins (15) identified in 2 of 3 chronic pancreatitis specimens and not in the other two cohorts, as illustrated in the Venn diagram in Figure 2. The number of peptides (#pep) identified and the sequence coverage (%cov) of each protein are indicated.

Table 3

Name	IPI #	CP1		CP2		CP3	
		#pep	%cov	#pep	%cov	#pep	%cov
cDNA FLJ60058, similar to Myosin light chain 1	IP100909366	2	15.7	2	36.8		
COL14A1 Isoform 2 of Collagen α1(XIV)	IP10050918			2	12.8	2	10.8
COL6A3 Isoform 1 of Collagen α3(VI)	IP100022200	3	15.4	6	18	8	27.3
DEFA1:LOC728358 Neutrophil defensin 1	IP100005721 *	4	34	7	36.2	3	67
FLNA Isoform 1 of Filamin-A	IP100333541			10	26.5	33	39.1
HIST2H2BF histone cluster 2, H2bf isoform b	IP100930174 *	9	87.3	8	84.3	12	93.3
HNRNPA1, nuclear ribonucleoprotein A1	IP100909288	3	66.1	2	41.7		
IGHA1 cDNA FLJ14473 fis, SNC73 protein (SNC73) mRNA	IP100386879			11	36.8	2	22.9
IGK@ IGK@ protein	IP100784985	1	28.5	6	48.5		
LMNA Progerin	IP100644087 *	1	12.9	3	13.5	5	23.3
MEAP2 microfilament-associated protein 2 isoform b	IP100644827			2	17	1	18.1
PFN1 Profilin-1	IP100216691			2	44.3	2	49.3
PKM2 Pyruvate kinase	IP100909560			6	39.9	2	27.4
RNASE3 Eosinophil cationic protein	IP100025427	2	37.5	2	48.1		
RPS8 Ribosomal protein S8	IP100645201	2	30.9			2	39.9

Abbreviations: NP, normal pancreas; CP, chronic pancreatitis; PC, pancreatic cancer.

* denotes proteins appearing in all 3 specimens.

Proteins (21) identified in 2 of 3 pancreatic cancer specimens and not in the other two cohorts, as illustrated in the Venn diagram in Figure 2. The number of peptides (#pep) and sequence coverage (%cov) identified in each specimen are indicated for each protein.

Table 4

Name	IPI #	PC1		PC2		PC3	
		#pep	%cov	#pep	%cov	#pep	%cov
ANXA4 Annexin A4	IP000872780			7	40.4	3	22.3
CALM3;CALM1;CALM2 cDNA, FLJ96792, similar to calmodulin 2	IP000916768			2	32.9	1	18.1
COL1A2 Collagen α2(I) chain	IP000304962			7	25.9	8	27.8
FN1 fibronectin 1 isoform 2 preproprotein	IP000845263	14	18.4			5	13.9
HBD;HBB Hemoglobin subunit delta	IP000473011 *	10	73.5	12	75.5	12	78.9
HIST1H1C Histone H1.2	IP000217465	12	51.6	16	67.1		
HNRNPA2B1 Putative uncharacterized protein HNRNPA2B1	IP000916517 *	3	34.1	4	46.1	2	45.1
HNRNPM Isoform 2 of nuclear ribonucleoprotein M	IP000383296			5	28.1	2	37.5
LOC100292021 similar to thioredoxin peroxidase	IP000938009			4	29.6	2	28.8
LYZ Lysozyme C	IP000019038			3	24.3	1	16.2
MYH9 Isoform 1 of Myosin-9	IP000019502	1	12.5			1	16.3
RPL14 60S ribosomal protein L14	IP000925323	1	23.9			2	38
RPL15;RPL15P3 Ribosomal protein L15	IP000550032			5	62.3	4	50.5
RPL21P119 hypothetical protein isoform 1	IP000887074 *	3	42.5	5	46.9	3	34.4
RPL23 10 kDa protein	IP000795751 *	3	86.8	9	71.4	3	81.3
RPL24 60S ribosomal protein L24	IP000306332	1	30.6	5	63.7		
RPL4 60S ribosomal protein L4	IP000003918 *	4	43.3	16	64.4	8	54.6
RPS7 40S ribosomal protein S7	IP000013415	1	26.8	5	36.1		
TKT cDNA FLJ54957, similar to Transketolase	IP000643920			5	28.5	1	15.9
VCL Isoform 2 of Vinculin	IP000307162			1	5.6	1	6.3
YWHAH Isoform Short of 14-3-3 protein beta/alpha	IP000759832	3	32.4			2	14.8

Abbreviations: NP, normal pancreas; CP, chronic pancreatitis; PC, pancreatic cancer.

* denotes proteins appearing in all 3 specimens.